Production and characterization of a tributyrin esterase from *Lactobacillus plantarum* suitable for cheese lipolysis

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ABSTRACT

Lactobacillus plantarum is a lactic acid bacterium that can be found during cheese ripening. Lipolysis of milk triacylglycerols to free fatty acids during cheese ripening has fundamental consequences on cheese flavor. In the present study, the gene *lp_1760* encoding a putative esterase/lipase was cloned and expressed in *Escherichia coli* BL21 (DE3) and the overproduced Lp_1760 protein has been biochemically characterized. Lp_1760 hydrolyzed *p*-nitrophenyl esters of fatty acids from C2 to C16, with preference for *p*-nitrophenyl butyrate. On triglycerides, Lp_1760 showed higher activity on tributyrin than on triacetin. Although optimal conditions for activity were 45 ºC and pH 7, Lp_1760 retains activity under conditions commonly found during cheese-making and ripening. Lp_1760 showed more than 50% activity at 5 ºC and exhibited thermal stability at high temperatures. Enzymatic activity was strongly inhibited by SDS and PMSF. Lp_1760 tributyrin esterase showed high activity in the presence of NaCl, lactic acid, and calcium chloride. The results suggest that Lp_1760 might be a useful tributyrin esterase to be used in cheese manufacture.

Key words: lipolysis, cheese ripening, aroma, triacylglycerols, lactic acid bacteria

INTRODUCTION

The characteristic flavor of cheese is the result of the breakdown of protein, fat and carbohydrates by milk native enzymes, added enzymes, starter bacteria and the secondary microbiota of cheese (Fox and Wallace, 1997). Lipolysis of milk triacylglycerols to free fatty acids during cheese ripening is an important biochemical process that contributes directly to its sensory characteristics (Collins et al., 2003). Free fatty acids are important sensory compounds by themselves, and also as precursors of other volatiles and esters (McSweeney and Sousa, 2000). The release of free fatty acids during cheese ripening by the combined action of lipolytic enzymes contributes to the development of flavor. Lipolytic enzymes include lipases, which catalyze the hydrolysis of water-insoluble long-chain triglycerols. The contribution of the native milk lipase to lipolysis in cheese is significant only in these varieties produced with raw milk, as this enzyme is inactivated during heat treatment. In other varieties, like Cheddar cheese, only a low level of milk fat hydrolysis occurs due to the weak lipolytic activities of the starter and non-starter bacteria (Crow et al., 1994).

Lactic acid bacteria are a source of esterase and lipases. Enzymes from *Lactococcus lactis* (Tsakalidou and Kalantzopoulos, 1992; Holland and Coolbear, 1996; Fernández et al., 2000), *Lactobacillus casei* (Castillo et al., 1999), *Lactobacillus fermentum* (Gobbetti et al., 1997), *Streptococcus thermophilus* (Liu et al., 2001), and *Micrococcus* sp. (Fernández et al., 2004) have been purified and characterized. Also, esterases and lipases from *Lactobacillus plantarum* have been partially purified (Anderson et al., 1995), purified (Gobbetti et al., 1996; Gobbetti et al., 1997; Oterholm et al., 1972; Silva Lopes et al., 2002) or recombinantly produced (Benavente et al, 2013; Brod et al., 2010; Esteban-Torres et al., 2013; Navarro-González et al., 2013). Among the lipases

described from *Lb. plantarum* (Anderson et al., 1995; Otherholm et al., 1967; Otherholm et al., 1968; Gobbetti et al., 1996; Gobbetti et al., 1997; Silva Lopes et al., 1999; Silva Lopes et al., 2002) none of them have been genetically identified so far.

The genome sequence of *Lb. plantarum* WCFS1 was published in 2003 (Kleerebezem et al., 2003) and more than twenty putative esterase or lipase genes were annotated on the basis of similarity searches. Although an operational distinction is made between esterases, which preferentially break the ester bonds of shorter chain acyl substrates at least partly soluble in water, and lipases, which display maximal activity toward water-insoluble long-chain triglycerides, there is no fundamental biochemical difference (Bornscheuer, 2002). From a structural viewpoint, both esterases and lipases are members of the α/β hydrolase superfamily, and share common catalytic machinery for ester hydrolysis and formation (Bornscheuer, 2002). Classifications based on sequence similarities do not separate the two classes of enzymes. The definitive approach to assigning a specific molecular function to a predicted open reading frame is to biochemically characterize the corresponding protein.

In this regard, the objective of this study was to determine the functional features of the putative esterase/lipase Lp_1760 from *Lb. plantarum* WCFS1, through biochemical characterization of the recombinantly expressed protein. With a view to applying this esterase under conditions found during cheese-making and ripening, enzyme activity under physicochemical conditions frequently encountered in cheese was studied.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth media, and materials

Lb. plantarum WCFS1 used through this study was kindly provided by M. Kleerebezem (NIZO Food Research, The Netherlands). *Escherichia coli* DH10B was used as host strain for all DNA manipulations. *E. coli* BL21 (DE3) was used for heterologous expression in the pURI3-Cter vector (Curiel et al., 2011). The *Lb. plantarum* strain was grown in MRS medium (Pronadisa, Spain) adjusted to pH 6.5 and incubated at 30 ºC. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 ºC and shaking at 200 rpm. When required, ampicillin was added to the medium at a concentration of 100 µg/mL.

Plasmid DNA was extracted by a High Pure plasmid isolation kit (Roche). PCR product was purified with a QIAquick gel extraction kit (Quiagen). Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). *Dpn*I and HS Prime Star DNA polymerase were obtained from TaKaRa. His-tagged protein was purified by a Talon Superflow resin (Clontech).

Lp_1760 gene cloning and expression

Genomic DNA from *Lb. plantarum* WCFS1 was extracted. The gene encoding a putative lipase/esterase (*lp_1760*) in *Lb. plantarum* WCFS1 was amplified by PCR by using the primers 1220 (5´-*TAACTTTAAGAAGGAGATATACATatg*atcaaagttactaaccgacact) and 1221 (5´-*GCTATTAATGATGATGATGATGATG*atttaaataataatcgaagaaatt) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *lp_1760* gene sequence are written in lowercase letters). Prime Star HS DNA polymerase (TaKaRa) was used for the PCR amplification. The 753-bp purified PCR product was inserted into the pURI3-Cter vector using a restriction enzyme- and ligation-free cloning strategy (Curiel et al., 2011). The vector produces recombinant proteins having a six-

116 histidine affinity tag in their C-termini. *E. coli* DH10B chemically competent cells were
117 transformed, recombinant plasmids were isolated, and those containing the correct insert
118 were identified by size, verified by DNA sequencing, and then transformed into *E. coli*
119 BL21 (DE3) cells for expression.
120
121 ***Lp_1760 protein purification***
122
123 Protein expression of the *lp_1760* gene was made using *E. coli* BL21(DE3) cells as
124 host strain. Cells carrying the recombinant plasmid pURI3-Cter-1760, were grown at 37
125 ºC in LB medium containing 100 μg mL^{-1} ampicillin on a rotary shaker (200 rpm) until an
126 optical density (OD) at 600 nm of 0.4 was reached. Isopropyl-β-D-thiogalactopyranoside
127 (IPTG) was added to a final concentration of 0.4 mM and protein induction was continued
128 at 22 ºC for 18 h. The induced cells were harvested by centrifugation (8,000 *g*, 15 min, 4
129 ºC). The cells were resuspended in phosphate buffer (50 mM, pH 7) containing 300 mM
130 NaCl and disrupted by French Press passages (three times at 1,100 psi). The insoluble
131 fraction of the lysate was removed by centrifugation at 47,000 *g* for 30 min at 4 ºC, and
132 the supernatant was filtered through a 0.2 μm pore-size filter and then loaded onto a Talon
133 Superflow resin (Clontech). The resin was equilibrated in phosphate buffer (50 mM, pH 7)
134 containing 300 mM NaCl and 10 mM imidazole to improve the interaction specificity in
135 the affinity chromatography step. The bound enzyme was eluted using 150 mM imidazole
136 in the same buffer. The purity of the enzyme was determined by SDS-PAGE in Tris-
137 glycine buffer. Fractions containing the His6-tagged protein were pooled, dialyzed against
138 phosphate buffer (50 mM, pH 7) containing 300 mM NaCl, and analyzed for esterase
139 activity.
140

Enzyme assay

Esterase activity was determined by a spectrophotometric method as described previously (Esteban-Torres et al., 2013) however *p*-nitrophenyl butyrate (Sigma-Aldrich) was used as substrate.

Substrate specificity, optimum pH and temperature, and thermostability

To investigate the substrate specificity of Lp_1760, activity was determined using different *p*-nitrophenyl esters of various chain lengths (Sigma-Aldrich): *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl caprylate (C8), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14) and *p*-nitrophenyl palmitate (C16) as substrates as described previously (Esteban-Torres et al., 2013).

The enzymatic activity of purified protein was also assayed against an ester library, which includes triacylglycerols, such as triacetin, and tributyrin (Esteban-Torres et al., 2013). *p*-Nitrophenol was used as pH indicator to monitor ester hydrolysis colorimetrically. The screening was performed in a 96-well Flat Bottom plate (Sarstedt) as described previously (Esteban-Torres et al., 2013).

Esterase activity was assayed in a pH range from 3.0 to 9.0, and at temperatures of 5, 20, 30, 37, 40, 45, 50, 55, 60, and 65 ºC as described previously (Esteban-Torres et al., 2013).

Effects of additives on Lp_1760 esterase activity

The effect of metals and ions on the activity of the esterase was assayed by incubation of the enzyme in the presence of each additive at a final concentration of 1 mM during 5 min at room temperature. Then, the substrate was added and the reaction was incubated at 37 ºC (Esteban-Torres et al., 2013). The compounds analyzed were $MgCl_2$, KCl, $MnCl_2$, $CuCl_2$, $NiCl_2$, $CaCl_2$, $HgCl_2$, $ZnCl_2$, DEPC, Cysteine, SDS, DTT, Triton X-100, Urea, Tween 80, Tween 20, EDTA, DMSO, PMSF and β-mercaptoethanol.

In addition, the effect of the presence of several compounds present during cheese-making and ripening was assayed. The effect of NaCl and $CaCl_2$ was determined by adding NaCl at concentrations ranging from 0 to 25% (w/v) or $CaCl_2$ from 0 to 10% (w/v). Reaction mixtures were prepared as described for the temperature optimum experiments, but different volumes of 25% NaCl or $CaCl_2$ were added, and the volume of the buffer was adjusted accordingly to maintain the final reaction volume. Reactions mixtures were pre-incubated for 5 min at room temperature before the enzyme was added. After the reaction, the absorbance was measured. The effect of the presence of lactic acid was assayed at concentrations ranging from 0 to 5 g/L. Reaction mixtures were prepared by adding different volumes of the corresponding stock solution (25 g/L).

RESULTS AND DISCUSSION

Production and characterization of Lp_1760 esterase

Lb. plantarum is a flexible and versatile bacterial species that is encountered in a wide variety of environmental niches, including some dairy, meat, and plant fermentations. The ecological flexibility of *Lb. plantarum* is clear by the observation that this species has one of the largest genomes known among LAB (Kleerebezem et al.,

2003). This large genome codifies enzymatic activities which could develop a fundamental role in food fermentations, such as esterases or lipases. When the reported sequence of the *Lb. plantarum* WCFS1 genome was analyzed, numerous ORFs encoding putative esterases/lipases were found. One of the first ORFs present in all the available *Lb. plantarum* genomes is *lp_1760*, predicted to encode a 250 amino acid sequence protein with a theoretical molecular mass of 28.8 kDa. The deduced amino acid sequence of Lp_1760 lacked a N-terminal secretion signal sequence suggesting that this enzyme is located intracellularly. The amino acid sequence of Lp_1760 showed a 56% identity to a putative lipase/esterase from *Lactobacillus sakei* 23K (accession Q38VR0) or a 52% identity to a hydrolase from *Enterococcus faecalis* ATCC 6055 (accession R3KRQ5 : data not shown).

The *lp_1760* gene was cloned into the pURI3-Cter expression vector by a ligation –free cloning strategy described previously (Curiel et al., 2011). The vector incorporates the DNA sequence encoding hexa-histidine to create a His-tagged fusion enzyme for further purification steps. The *lp_1760* gene was expressed under the control of an IPTG-inducible promoter. Cell extracts were used to detect the presence of overproduced proteins by SDS-PAGE analysis. An overproduced protein, with an apparent molecular mass around 28 kDa was apparent in cells harbouring pURI3-Cter-1760 (Figure 1). The recombinant protein was purified by a metal affinity chromatography (IMAC) resin, and eluted with phosphate buffer (50 mM, pH 7) containing 300 mM NaCl and 150 mM imidazole. The eluted His6-tagged Lp_1760 was dialysed, and observed as a single band on SDS-PAGE (Figure 1). Routinely, the yield of purification is about 8 mg/L of purified protein.

Lp_1760 purified protein was biochemically characterized. Substrate specificity was determined using *p*-nitrophenyl-linked esters of various acyl chain lengths (C2 to

215 C16) at 37 ºC (Figure 2). Lp_1760 showed activity on all the acyl esters assayed,
216 exhibiting significant activity on *p*-nitrophenyl palmitate (C16). The observed activity on
217 long chain acyl esters confirmed that Lp_1760 is a true lipase. For the sake of comparison,
218 we have determined spectrophotometrically the kinetic parameters for the most reliable
219 substrates C2 and C4. In both cases, Lp_1760 exhibited a hyperbolic Michaelis-Menten
220 kinetics (not shown). The kinetic parameters are shown in Table 1. From the values of
221 these parameters it can be deduced that the catalytic efficiency (k_{cat}/K_m) for *p*-nitrophenyl
222 butyrate hydrolysis is around 2-fold the one observed for pNPA hydrolysis, which is due
223 to a lower K_M for *p*-nitrophenyl butyrate since k_{cat} is essentially the same.
224 Esterase activity was revealed when the hydrolysis of esters present in the library
225 were assayed. Lp_1760 showed highest activity on tributyrin although in triacetin 30% of
226 the tributyrin activity was observed (data not shown). Apart from tributyrin and triacetin,
227 from the 40 esters assayed, only methyl hydroxyacetate, phenyl acetate, and vinyl
228 octanoate, were minimally hydrolyzed (data not shown). Tributyrin is a true fat and the
229 simplest triglyceride occurring in natural fats and oils. The principal lipids of milk are
230 triacylglycerides, which may represent up to 98% of the total lipids (Collins et al., 2003).
231 Triacylglycerides are esters of glycerols composed of a glycerol backbone with three fatty
232 acids attached. Tributyrin is a common constituent of lipase testing media as it is easily
233 dispersed in water (Samad et al., 1989). Tributyrin esterases from *Lb. plantarum* have
234 been isolated and partially purified previously (Anderson et al., 1995; Gobbetti et al.,
235 1996; Gobbetti et al., 1997; Silva Lopes et al., 2002) however they have not been
236 genetically identified so far.
237 Regarding the dependence on pH of the hydrolytic activity, the lipase was active
238 within the range comprised between 3 and 8, exhibiting an optimal pH around 7 (Figure
239 3A). Lipases from *Lb. plantarum* 2739 exhibited a similar optimal pH (7-7.5) (Gobbetti et

al., 1996; Gobbetti et al., 1997), whereas the purifed *Lb. plantarum* MF32 lipase exhibited an optimal alkaline pH (pH 9.3; Anderson et al., 1995), or the extracellular lipase from *Lb. plantarum* DSM 12028 which showed an optimal acidic pH (pH 5.5) (Silva Lopes et al., 1999).

The influence of temperature on enzymatic activity was also determined (Figure 3B). The *L. plantarum* lipases characterized so far have optimal temperatures at 30 ºC (Silva Lopes et al., 1999), 35 ºC (Gobbetti et al., 1996; Gobbetti et al., 1997) or 37 ºC (Anderson et al., 1995). Contrarily, maximal activity of Lp_1760 was observed at 45 ºC. More interestingly, Lp_1760 exhibited more than 50% of the maximal activity at 5 ºC, and the activity only decreases to 40% at 65 ºC. The high esterase activity observed within this broad range of temperatures makes this lipase a good candidate for industrial applications. Note that thermophilicity is related to the capacity of the enzyme to hydrolyze the substrate at high temperatures, while thermal stability is defined as an enzyme ability to resist thermal unfolding in the absence of its substrate (Dotsenko et al., 2012). Figure 3C showed that Lp_1760 exhibited high thermal stability under prolonged incubation at temperatures up to 45 ºC (Figure 3C).

The effects of several ions and additives on Lp_1760 activity are provided in Table 2. Compared to the enzyme incubated in 50 mM phosphate buffer pH 7, the enzymatic activity was not significantly increased by any of the ions or additives assayed. However, Lp_1760 activity was significantly inhibited by Hg^{2+}, Zn^{2+} and Cu^{2+} ions; and greatly inhibited by SDS or PMSF. Inactivation of the tributyrin esterase by PMSF suggests that a serine residue is involved in the catalytic mechanisms of the enzyme. This is highly probable as most of the esterases and lipases have been previously characterized as having a Ser-Asp-His catalytic triad (Bornscheuer, 2002).

265 ***Activity of Lp_1760 in the presence of conditions found during cheese-making and***
266 ***ripening***
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268 Unlike many processed food products for which stability is the key criterion,
269 cheese is a biochemically dynamic product and undergoes significant changes during its
270 ripening period. Lipolysis, or rather the extent to which it occurs during cheese ripening, is
271 an important factor in the development of the correct and characteristic flavour profile of
272 most cheese varieties. The use of exogenous lipases, microbial among them, has been
273 reported in the scientific literature for the manufacture of a variety of cheese to accelerate
274 ripening or to develop characteristic flavors (Law, 2001; Hernández et al., 2005).
275 In order to ascertain the possible usefulness of Lp_1760 lipase during cheese-
276 making and ripening, the influence on its activity of the conditions and compound present
277 on these processes was studied. Cheese-making processes subject microorganisms, and
278 their enzymes, to adverse environmental conditions (such as acid or osmotic stress) which
279 affect their technological performances. Lowering the pH in lactic acid fermentations may
280 reduce the activity or completely inactivate enzymes that could generate either flavour
281 components or flavor precursor compounds. The pH of cheese is controlled by the
282 interactive effects of a number of critical factors including the amount of lactic acid,
283 calcium phosphate and proteins, the salt sensitivity of the starter culture, and the level and
284 duration of salting (Hou et al., 2012). Figure 3 indicates that Lp_1760 exhibited 40% of its
285 maximal activity at the pH usual during cheese-making or ripening (pH near 5).
286 In relation to temperature, cheese ripening temperature is characteristic of each
287 cheese variety. As an example, Cheddar-type cheeses may be cured or ripened at up to 15
288 ºC for a period of several months, whereas Swiss-type cheeses are cured at 22-23 ºC. The
289 ripening temperature could be different from the storage or distribution temperature. It is

recommended that pasteurized milk cheeses with <50% moisture, with traditional levels of salt, starter culture, pH, and fat be allowed to be distributed at a temperature not exceeding 30 ºC (Johnson et al., 2009). At 15-30 ºC, Lp_1760 showed 50-70% of its maximal activity, which makes it adequate for these processes.

Due to its industrial relevance, a better knowledge of the influence of compounds present during cheese-making or ripening is important. The influence on Lp_1760 activity by compounds present in these processes (such as NaCl, lactic acid, or calcium chloride) was also studied.

One of the final steps in the cheese-making process is the addition of salts. In dry salted cheese like Cheddar, the salt is added directly to the curds just before hooping and pressing. In other types of cheese, the salt is added by submersing the cheese in brine (up to 20% NaCl) for an appropriate period of time and salt penetrates slowly during brining and the subsequent storage period. Finally, some cheeses have salt rubbed on the outer side of the cheese (Johnson et al., 2009). The influence of NaCl on Lp_1760 activity has been analyzed. Salt concentrations up to 10% increases lipase activity (Figure 4A). Contrarily, concentrations higher than 10% partially inhibited esterase activity, however, Lp_1760 retained half of the maximal activity at 25% NaCl concentration. At salt concentrations usual on final cheeses, the activity of Lp_1760 is increased.

In relation to lactic acid, starter bacteria are used to ferment the lactose to lactic acid. The rate and extent of acid development is controlled by the cheese maker to produce the final desired body, texture, and flavor characteristics of the cheese. Total lactate levels increased significantly with ripening time. The increase in lactate content with ripening time is consistent with the decrease in lactose levels, which is metabolized to lactic acid during ripening by starter and non-starter bacteria. Lactate content from 1 to 1.5% (w/w)

has been described during ripening of Cheddar cheese (Hou et al., 2012). At these lactic acid concentrations, Lp_1760 exhibited fully enzymatic activity (Figure 4B).

Calcium chloride is usually added to cheese-milk during cheese-making to assist coagulation, improve the cheese-making process, and/or increase the yield. The normal range of calcium addition spanning from 0 to 0.5 g/L (Ong et al., 2013). Calcium chloride concentrations up to 0.1 g/L (1%) did not affect Lp_1760 activity (Figure 4C). The activity showed by Lp_1760 suggests that this esterase could play a role in modulating flavor profile during cheese ripening.

The obtained results indicated that Lp_1760 is a tributyrin esterase that retains activity under conditions commonly found during cheese-making and ripening, such as cold temperature, acidic pH, and the presence of salt, lactic acid or calcium chloride. Another advantage for the potential use of Lp_1760 in cheese-making is its thermostability. Further assays need to be done under combined conditions to further evaluate the effect of the simultaneous presence of different factors on Lp_1760 activity.

CONCLUSIONS

In the present study, the tributyrin esterase Lp_1760 from *Lb. plantarum*, an important non-starter LAB species which can be found during cheese ripening, was purified and biochemically characterized. Lp_1760 tributyrin esterase may contribute positively to cheese flavour development as it shows marked activity on tributyrin and on *p*-nitrophenyl esters of fatty acids. Lp_1760 exhibits high activity at the conditions (temperature and pH) and in the presence of compounds (salt, lactic acid or calcium chloride) found during cheese-making and ripening. Based on the findings reported in this

study, a tributyrin esterase/lipase of such characteristics may play a relevant role in flavour development during ripening, either produced in situ or added to milk or curd cheese manufacture.

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446 extracellular esterase from *Lactococcus lactis* subsp. *lactis* strain ACA-DC 127.
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448
449
450 **FIGURE CAPTIONS**
451
452 **Figure 1**. SDS-PAGE analysis of the purification of Lp_1760 tributyrin esterase from *L.*
453 *plantarum* WCFS1. Analysis by SDS-PAGE of soluble cell extracts of IPTG-induced *E.*
454 *coli* BL21(DE3) (pURI3-Cter) (1) or *E. coli* BL21(DE3) (pURI3-Cter-1760) (2),
455 flowthrough (3), or fractions eluted after His affinity resin (4-7). The arrow indicated the
456 overproduced and purified protein. The gel was stained with Coomassie blue. Molecular
457 mass markers are located at the left (SDS-PAGE Standards, Bio-Rad).
458

459 **Figure 2**. Substrate profile of Lp_1760 against chromogenic substrates (*p*-nitrophenyl
460 esters) with different acyl chain lengths (C2, acetate, C4, butyrate, C8, caprylate, C12,
461 laurate, C14, myristate, C16, palmitate). The figure displays the relative specificities
462 obtained toward different substrates, and lines on top of each bar represent the standard
463 deviations estimated from three independent assays. The observed maximum activity was
464 defined as 100%.
465
466 **Figure 3**. Biochemical properties of Lp_1760 tributyrin esterase. (A) pH-activity profile
467 of Lp_1760. (B) Temperature-activity profile of Lp_1760. (C) Thermal stability profile for
468 Lp_1760 after preincubation at 22 ºC (filled diamond), 30 ºC (filled square), 37 ºC (filled
469 triangle), 45 ºC (cross), 55 ºC (star), and 65 ºC (filled circle) in phosphate buffer (50 mM,
470 pH 7), at indicated times, aliquots were withdrawn, and analyzed as described in the
471 Materials and Methods section. The experiments were done in triplicate. The mean value
472 and the standard error are indicated. The percentage of residual activity was calculated by
473 comparing with unincubated enzyme.
474
475 **Figure 4.** Activity of Lp_1760 esterase in the presence of compounds found during
476 cheese-making process. Relative activity of Lp_1760 after incubation in the presence of
477 sodium chloride (A), lactic acid (B), and calcium chloride (C) at the concentrations
478 indicated. The activity of the enzyme in the absence of the compound was defined as
479 100%. The experiments were done in triplicate. The mean value and the standard error are
480 shown.

Table 1. Kinetic parameters for pNPA and pNPB hydrolysis by tributyrin esterase.

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/ K_M (s^{-1} mM^{-1})
pNPA	0.60 ± 0.18	2.17 ± 0.86	0.30 ± 0.11	0.14 ± 0.05
pNPB	0.46 ± 0.09	0.95 ± 0.30	0.23 ± 0.06	0.24 ± 0.09

Enzyme activities were determined at 45 ºC in 50 mM sodium phosphate buffer, pH 7.0. Results are the mean value ± SD from three independent experiments.

Table 2

Table 2. Effect of additives on Lp_1760 tributyrin esterase activity

Additions (1 mM)	Relative activity (%)
Control	100
KCl	89
$HgCl_2$	40
$CaCl_2$	106
$MgCl_2$	105
$ZnCl_2$	59
$CuCl_2$	51
$NiCl_2$	92
$MnCl_2$	90
Tween 20	94
Tween 80	78
Triton X-100	100
SDS	11
Urea	115
DMSO	97
Cysteine	109
DTT	101
β-mercaptoethanol	90
EDTA	101
PMSF	17
DEPC	97

Figure 1

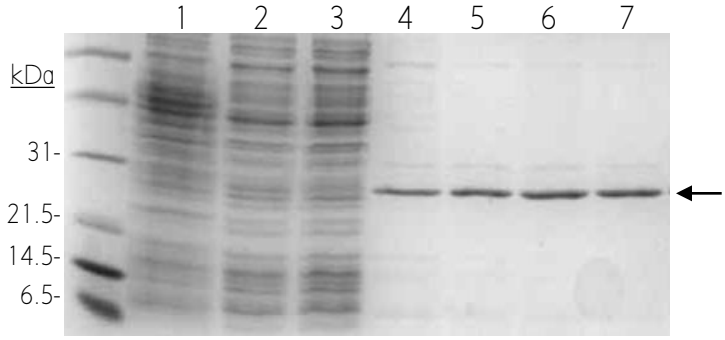


Figure 2

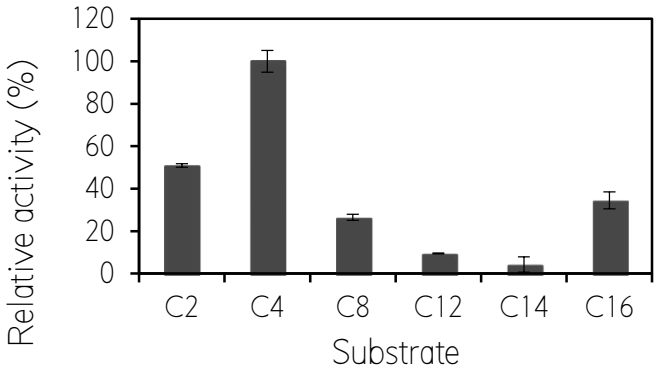

120
100
80
60
40
20
0
Relative activity (%)
C2
C4
C8
C12
C14
C16
Substrate

Figure 3

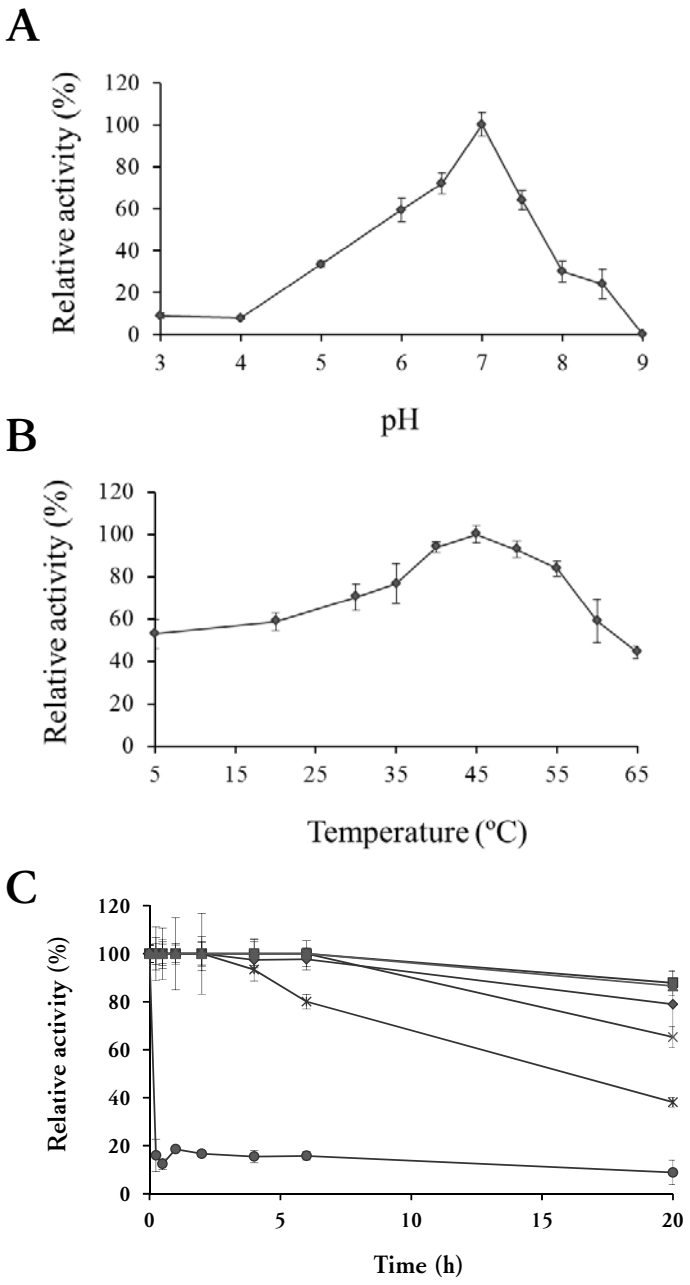
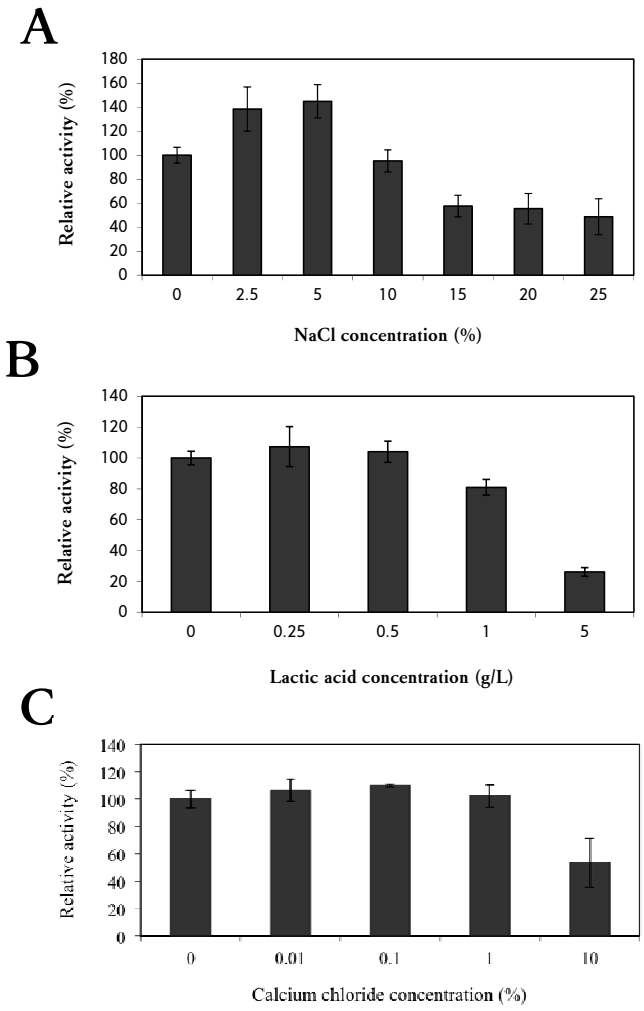

A
Relative activity (%)
120
100
80
60
40
20
0
3
4
5
6
7
8
9
pH
B
Relative activity (%)
120
100
80
60
40
20
0
5
15
25
35
45
55
65
Temperature (°C)
C
Relative activity (%)
120
100
80
60
40
20
0
0
5
10
15
20
Time (h)

Figure 4


A
Relative activity (%)
180
160
140
120
100
80
60
40
20
0
0
2.5
5
10
15
20
25
NaCl concentration (%)
B
Relative activity (%)
140
120
100
80
60
40
20
0
0
0.25
0.5
1
5
Lactic acid concentration (g/L)
C
Relative activity (%)
140
120
100
80
60
40
20
0
0
0.01
0.1
1
10
Calcium chloride concentration (%)